ORIGINAL

Adsorption of mercury and copper ions by oral microorganisms

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Abstract : The metal ion accumulation by oral bacterial cells was investigated in this study using two heavy metals, copper and mercury at pH 4.0 and 7.0. However, the adsorption rate of mercury was especially examined at pH 4.0 because of the loss of metal ions caused by the adsorption on the walls of the container and the occurrence of volatilization at pH value on the neutral and alkaline side.

Higher mercury adsorption by bacterial cells were demonstrated in *Propionibacterium acnes*, *Treponema denticola* and *Eikenella corrodens* in order of strength at pH 4.0. Regarding copper ion adsorption by oral bacteria, the higher metal adsorptions were also shown at pH 4.0 and 7.0 in *P. acnes*, *Peptostreptococcus anaerobius* and *Corynebacterium matruchotii*, respectively.

The metal adsorptive abilities of these oral microorganisms appeared to be higher than that of *Actinomyces levoris* which had extremely high uranium accumulation abilities etc.

There were no marked differences between the intact cells and the lyophilized cells of *P. acnes*, even when treated with heat at 100°C for 10 min.

These metal ion adsorptions by microorganisms always proceeded immediately after adding metal to bacterial suspension and reached a plateau around 5 min later.

These findings obtained strongly suggest that some oral bacteria have the potential the adsorption of heavy metal ions, and that the mode of adsorption is based on a physico-chemical action governed by the Freundlich adsorption isotherm equation.

抄録 : 口腔細菌による金属イオンの吸着を、二つの重金属、水銀および銅について pH 4.0 と 7.0 域で調べた。しかしながら、二つの金属のうち、水銀イオンは、中性およびアルカリ域では、蒸発や容器壁への吸着のためイオンの損失が起こるので、pH 4.0 で吸着率を検討した。

高い水銀イオン吸着を示した菌種は、pH 4.0 域の際に、供試菌の中で、*Propionibacterium acnes*, *Treponema denticola* および *Eikenella corrodens* であった。銅イオンの吸着については、pH 4.0 と 7.0 域において、*P. acnes*, *Peptostreptococcus anaerobius* および *Corynebacterium matruchotii* が高い吸着を示した。

これらの口腔細菌の金属イオン吸着能は、同じ金属イオン濃度では、従来ウラン等を高率に吸着すると報告されている *Actinomyces levoris* よりも高い値を示した。また、100 度で 10 分間加熱処理した凍結乾燥菌体への銅イオンの吸着は、無処理の菌体と比較して、著しい差異を示さなかった。

また、細菌への金属イオン吸着は、金属イオンの添加直後に起こり、5 分後にはプラトーに達した。

得られた成績は、いくつかの口腔細菌が金属イオンの高い吸着能を有すること、また、これらの吸着現象は、Freundlich の吸着等温式に従っていることから物理化学的作用によると考えられる。

Introduction

More than twenty heavy metals have widely been applied and used as restorative materials such as amalgams and alloys in dental clinics¹⁾. Most are thought to be non-toxic but a few sometimes exhibit toxicity to the human body²⁾.

It has been suggested that several heavy metal elements might be released from amalgams and alloys resulting in toxicity and allergenicity³⁾.

On the other hand, it is recognized that many kinds of heavy metals are capable of being adsorbed and accumulated to biomass such as bacteria, algae, fungi, yeast and even dead cells and their products⁴⁻⁸⁾. In the oral cavity, many kinds of oral bacteria are able to colonize on the teeth, in subgingival crevices and gingival pockets to produce supra and subgingival plaque. This dental plaque sometimes results in dental caries and periodontal diseases associated with carbohydrate ingestion. Considering such points most dentists have thought that the plaque should be removed by brushing and flossing. On the contrary, it may be presumed that dental plaque is a sort of bacterial film and acts as a barrier in defence of the oral ecosystem.

However, the intrinsic biological role of these normal flora is still not fully known.

The purpose of this investigation was to demonstrate and elucidate the heavy metal ion-adsorption by the oral flora. The adsorbing characteristics of two heavy metals, mercury and copper were studied.

Materials and Methods

Organisms

Ten strains, *Actinomyces levoris* HUT 6156, *A. naeslundii* ATCC 12104, *Corynebacterium matruchotii* ATCC 14622, *Eikenella corrodens* ATCC 10596, *Fusobacterium nucleatum* ATCC 10539, *Peptostreptococcus anaerobius* ATCC 27377, *Porphyromonas gingivalis* ATCC 33277, *Propionibacterium acnes* ATCC 6919, *Treponema denticola* ATCC 33520 and *Streptococcus mutans* LM-7 were subcultured and used for this experiment.

The microorganisms described above are listed in

Table 1. These microorganisms except *A. levoris* are easily isolated from the oral cavities of most individuals.

Metal ions

Two metals, copper and mercury were purchased from Nakalai Tesque Inc. Kyoto in an ionic state. Copper standard solution (stock solution, Cu 1,000 ppm, CuSO₄ in 0.1 mol of HNO₃) was diluted with distilled water and adjusted to pH 4.0 and 7.0 with 0.1N NaOH and 0.1N HNO₃ solution. Mercury standard solution (stock solution, Hg 1,000 ppm, HgCl₂ in water) was also diluted with distilled water and adjusted as above.

Culture

A. levoris was aerobically subcultured in a liquid medium consisting of 3 g meat extract, 5 g peptone and 5 g of NaCl per liter (pH 7.0) at 37°C for 24 hr. *A. naeslundii* was aerobically cultured in brain heart infusion (BHI) broth (BBL, Microbiology systems, pH 7.0) at 37°C for 24 hr. *C. matruchotii* and *S. mutans* were aerobically cultured in BHI broth supplemented with 0.2% yeast extract at 37°C for 24 hr. Several anaerobes, *P. anaerobius*, *P. acnes*, *E. corrodens* and *F. nucleatum* were anaerobically cultured in GAM (Gifu Anaerobic Medium, Nittsui, pH 7.0) at 37°C for 48 hr. *P. gingivalis* was also cultured in GAM supplemented with hemine (1%) and menadione (0.1%, pH 7.0). *T. denticola* was anaerobically cultured in TYGVS (trypticase soy, yeast extract, volatile, fatty acids, and serum, pH 7.2) at 37°C for 7 days⁹⁾.

Lyophilized cells

After cultivation, each bacterial cell was centrifuged at 6,000 g for 30 min, and washed 3 times with distilled water. The harvested cells were lyophilized, and kept in a freezer until use.

Viable cells

The *P. acnes* cells grown in GAM medium were immediately harvested, and washed with distilled water and centrifuged at 2,500 rpm. The cell concentration was adjusted with distilled water to make a final concentration of 7.37×10^{11} cells/ml by using a

bacterial cell counter (Petroff-Hausser, U. S. A.).

Heat-killed cells

Lyophilized cells : The cells of lyophilized *P. acnes* were treated in boiling water for 10 min. The cell concentration was adjusted with distilled water to make a final concentration of 1 mg/ml.

Viable cells : The cells of grown *P. acnes* were harvested and treated in the same manner as above. The cell concentration was adjusted with water to make a final concentration of 7.37×10^{11} cells/ml by using bacteria counter (Petroff-Hausser, U. S. A.).

Adsorption experiments

All glass test tubes and polypropylene tubes were immersed in 1N HNO₃ solution for 4hr and rinsed several times with deionized water before use to avoid metal contamination.

In the mercury adsorption test, the cells (20 mg dry weight) were suspended in 10 ml of distilled water. An amount of 0.5 ml bacterial suspension was mixed in 4.5 ml of Hg (a final concentration of 0.45 μ g/ml, 2.24 μ M) solution. In the copper adsorption test, an amount of 0.5 ml bacterial suspension was mixed in 2 ml of Cu (a final concentration of 1.6 μ g/ml : 25.2 μ M) solution. The pH of the aqueous solution was adjusted to pH 3.0 to 8.0 with 0.1M HNO₃ and 0.1M NaOH solution⁶⁾. After each suspension was incubated and stirred for 1hr at 37°C, each solution was centrifuged for 30 min at 23,000 g and the supernatant was collected. The amounts of free metal ions in the supernatants were determined by atomic adsorption spectrometer (Shimadzu AA660). The adsorption rate was determined as the following formula : the rate = (Initial metal concentration - Residual metal concentration in the solution) / Initial metal concentration in the solution $\times 100$.

Mercury assay

The mercury measurements were carried out with a flameless atomic adsorption spectrometer equipped with a cold vaporizer of mercury (MVU-1A, Shimadzu). The cold-vapor technique by Ebbestad U *et al*¹⁰⁾ was modified. With a syringe, 2.5 ml of a 10% SnCl₂ solution in 1N H₂SO₄ was injected through the se-

ptum. The apparatus was calibrated for each type of sample using acidified and stabilized mercury stock solution¹¹⁻¹³⁾.

Copper assay

The analysis for copper was carried out with an atomic adsorption spectrometer (Shimadzu AA660) over an acetylene flame¹⁴⁾.

Adsorption isotherm of metal ions by bacteria

Adsorption reaction has been characterized by an adsorption isotherm. Many isotherms of adsorption reaction have been proposed, but the Freundlich appeared to be most universally accepted.

The effect of the metal concentration in the solution on copper adsorption by *P. acnes* was examined. The lyophilized cells of *P. acnes* at a final concentration of 0.2 mg/ml was suspended and incubated with four different concentrations of copper (10 to 500 μ g/ml) at 37°C for 1hr, and the copper ions adsorbed by the cells were measured and estimated in relation to their concentration in the solution.

Statistical analysis

Analysis of variance for multiple comparisons was used for assessment of the statistical relevance of the results. The adsorption test was repeated 7 times on the recovery rate of the mercuric and copper ions.

Results

Mercury stability at various pHs

The recovery of the mercuric ions was markedly affected by the pH range (Fig. 1). At the pH values above 5 to 8, the mercury recovery rate was markedly reduced. However, at the pH 4.0 the recovery was about 100%. On the other hand, the recovery of copper ions was not markedly affected by the pH of the solution (data not shown). The mercury-adsorbing abilities were examined and compared with pH 4.0 and 7.0 range.

Also, the adsorption rate of copper ions was examined at both the 4.0 and the 7.0 range.

Fig. 1 Effect of pH on the recovery rate of mercuric ions from aqueous solution.

Fig. 2 Adsorption of copper ions by heated and non-heated cells of *P. acnes* at pH 7.0.

A : Viable cells harvested immediately after culture ; The heated cells of *P. acnes* were prepared by immersing the living cells in boiling water for 10 min. The concentrations of the cells used for adsorption test were adjusted to make a final concentration of 7.37×10^{11} cells/ml by using bacterial counter.

B : Lyophilized cells ; The heated cells of *P. acnes* were prepared in a same manner as above. The concentrations of the cells used for adsorption test were adjusted to 1 mg/ml by measuring dry weight.

□ : non-heated cells, ■ : heated cells

Fig. 3 Adsorption rate of mercuric and copper ions by heated and non-heated cells of lyophilized *P. acnes* at pH 4.0.

A : mercury, B : copper, ○ : non-heated cells, ● : heated cells

The heated-killed cells of *P. acnes* were prepared by immersing the lyophilized cells in boiling water for 10 min. The concentrations of the cells used for adsorption test were adjusted to make a final concentration of 1 mg/ml by measuring dry weight.

Adsorption of metal ions by various microorganisms

The adsorption rate of mercuric ions in each bacterial suspension of double dilution series was examined at pH 4.0 (Fig. 4). With a final concentration of the microorganisms at 0.1 mg/ml, the rate of adsorption by *P. acnes*, *T. denticola* and *E. corrodens* were about 90% and were most effective on the mercuric ions. The rate by *A. levoris* showed 70% and that by *C. matruchotii* was about 20%.

The rate of copper ion adsorption by various microorganisms at pH 4.0 is shown in Figure 5. With a final concentration of the microorganisms at 0.4 mg/ml, the rate by *T. denticola*, *P. anaerobius* and *C. matruchotii* were about 80%, and were most effective on copper ion adsorption. *A. levoris* and *P. acnes* showed the rate of about 75%. The rates of adsorption by

Fig. 4 Adsorption rate of mercuric ions by various microorganisms at pH4.

A. levoris (☆), *A. naeslundii* (△), *C. matruchotii* (■), *E. corrodens* (◇), *F. nucleatum* (★), *P. acnes* (●), *P. anaerobius* (×), *P. gingivalis* (○), *S. mutans* (◆), *T. denticola* (▲).

Each suspension was incubated with shaking for 1 hr at 37°C.

Adsorption rate = $100 \times (\text{Initial metal concentration} - \text{Residual metal concentration in the solution}) / \text{Initial metal concentration in the solution}$

Fig. 5 Adsorption rate of copper ions by various microorganisms at pH4. See footnote to Figure. 4. Agarose (□).

F. nucleatum, *A. naeslundii* and *P. gingivalis* were less than 50%.

The rate of adsorption of cupric ions by various microorganisms at pH 7.0 is shown in Figure 6. With a final concentration of microorganisms at 0.4 mg/ml, *P. acnes* was most effective on the adsorption of copper ions and was about 90%. The rates by *P. anaerobius*, *C. matruchotii* and *E. corrodens* ranged from 85% to 75%. However, the rate of adsorption by *A. levoris* was 70%, that by *P. gingivalis* and *T. denticola* were the lowest and below 40%. Agarose,

Fig. 6 Adsorption rate of copper ions by various microorganisms at pH7. See footnote to Figure. 4. Agarose (□).

Fig. 7 Time course of adsorption of mercuric and copper ions by *P. acnes* at pH4.
A : mercury, B : copper

which is used widely as a matrix in affinity chromatography, was used as the control of metal adsorbing-abilities⁸⁾. Copper adsorption by agarose was about 20% (Figs. 5, 6).

The mercuric ions-adsorbing ability differed with the various species of microorganisms at pH 4.0. About half the microorganisms took up more than 80% of the mercury in solution. Extremely high mercury-adsorbing ability was observed in *P. acnes*,

Table 1 List of microorganisms used including Gram stain and morphology.

Microorganisms	Gram stain	Morphology
<i>Actinomyces levoris</i> HUT 6156	+*	rod
<i>A. naeslundii</i> ATCC 12104	+	rod
<i>Corynebacterium matruchotii</i> ATCC 14622	+	rod
<i>Eikenella corrodens</i> ATCC 10596	-**	rod
<i>Fusobacterium nucleatum</i> ATCC 10539	-	rod
<i>Peptostreptococcus anaerobius</i> ATCC 27377	+	coccus
<i>Porphyromonas gingivalis</i> ATCC 33277	-	rod
<i>Propionibacterium acnes</i> ATCC 6919	+	rod
<i>Streptococcus mutans</i> M-7	+	coccus
<i>Treponema denticola</i> ATCC 33520	-	spiral

*+ : positive, ** - : negative

Fig. 8 Freundlich isotherm equation for adsorption of copper ions by *P. acnes*.

Adsorption experiment was carried out by suspending the cells (dry weight, 4 mg) in 2.5 ml copper solution (pH7.0).

T. denticola and *E. corrodens*.

Adsorption of metal ions by non-heat cells and heat killed cells

With the viable cells immediately after the culture of *P. acnes*, the adsorption rate of copper by the viable cells was decreased by about 6.4% by heat treatment at pH 7.0 (Fig. 2), but the rates of the decrease was insignificant.

On the other hand, there were no differences between the lyophilized cells and their heated cells regarding the amount of metal ions.

With the lyophilized cells, the rates of the two metal ions were not decreased by the heat treatment at pH 4.0 (Fig. 3). The mercuric and copper ions-adsorbing abilities of the heat-killed cells and non-heat lyophil-

ized cells were similar.

Time course of the adsorption of metal ions by *P. acnes*

The time course of the adsorption of mercuric and copper ions by *P. acnes* was examined at pH 4.0. The adsorption of mercuric and copper ions by *P. acnes* reached a plateau 5 min after addition of the metal ions and did not increase thereafter (Fig. 7). This rapid adsorption of metal ions by *P. acnes* was similar to that observed with *A. levoris*^{15).}

Adsorption of copper by *P. acnes* according to Freundlich isotherm

The adsorption experiment was carried out by suspending the cells (4 mg of dry weight) in 2.5 ml of copper solution at pH 7.0, to make four dilution series. The amount of copper adsorbed at these four concentration levels formed a straight line on a log-log graph (Fig. 8).

Statistical analysis

Significant differences between the mean control and mean experimental counts were determined by using the coefficient of variance. On both copper and mercury, the 95% confidence limit extended to 2.8% of the mean adsorption value with the means at 7 times. Therefore, standard errors were less than 2.8% in each case.

Discussion

The adsorption of mercuric and copper ions by oral microorganisms was studied. The assays were conducted under similar conditions as to pH and temperature in the environment of the oral cavity. The temperature was set at 37°C. However, the pH of the solution for mercury assay was set at 4.0 because mercury in general tends to volatilize at pH 7.0 but not at pH 4.0 (Fig. 1) thereby allowing sufficient recovery¹⁶⁾. On the other hand, the copper measurements were easily carried out at both pH 4.0 and 7.0 because of non-volatility. In this experiment the amounts of bacteria were estimated as the dry weight of the lyophilized cells. This is because there were almost no differences between the adsorbing abilities of the lyophilized and viable cells, and estimation by using the dry weight was easier than using a bacterial counter for the comparison of metal adsorption with another bacteria (Fig. 4~6).

The effects of the lyophilized cell concentrations of oral bacteria on metal adsorption were examined. At pH 4.0 with the concentration of the microorganisms at 0.1 mg/ml, mercury-adsorption higher than 80% was observed in *P. acnes*, *E. corrodens* and *T. denticola* (Fig. 4). Besides, with the final concentration of the microorganisms at 0.4 mg/ml, all *T. denticola*, *P. anaerobius* and *C. matruchotii* showed over 80% adsorption of copper adsorption in ratio (Fig. 5). At pH 7.0, copper-adsorption by *C. matruchotii*, *P. acnes* and *P. anaerobius* was observed exceeding 80% (Fig. 6).

It has been reported that the genus *Actinomyces* differs from many other bacteria and has a high adsorptive ability of uranium and mercury⁷⁾. However, the results obtained in these experiments indicated that the mercury-adsorbing abilities of *A. levoris* and *A. naeslundii* species were less than those of *E. corrodens*, *F. nucleatum*, *P. anaerobius* and *T. denticola* for mercury at pH 4.0 and of *C. matruchotii*, *P. anaerobius* and *T. denticola* for copper at pH 7.0. It appears that metal-adsorption may differ markedly among bacterial species.

These results suggest that the *Actinomyces* may not

be the only genus having high mercury and copper-adsorbing abilities.

Cells of these microorganisms were Gram-negative or positive, and were cocci or rods morphologically (Table 1). Like most cell surfaces, the bacterial cell wall is anionically charged¹⁷⁾. Most cell walls of Gram-positive bacteria contain relatively large amounts of peptidoglycan and an anionic polymer, such as a teichoic or a teichuronic acid. All walls of Gram-positive bacteria are negatively charged in media capable of supporting growth. On the other hand, the envelope of Gram-negative bacteria is, however, more complex than that of its Gram-positive counterpart.

It seems that there may be some differences in the structure of the cell walls between Gram-negative and-positive bacteria, as they effect metal-adsorbing abilities. However, the results obtained suggest that neither the differences of Gram stain ability nor of morphology are related to the metal-adsorption of microorganisms.

There were marked differences in the adsorbing-abilities between mercuric and copper ions by *C. matruchotii* (Fig. 4~6). In addition, the significant differences in the adsorption of copper ions by *T. denticola* at the two pH points between pH 4.0 and 7.0, were observed. However, the modes of adsorptive action remains unknown.

It has been previously reported that some elemental metals, nickel and mercury, etc. released from dental amalgam and alloys might exhibit toxicity to human bodies^{3,18)}, while, several researchers have pointed out that enough indirect evidence exists to assume the passivity of amalgams in the oral cavity. Thus reducing the risk of factors regarding of mercury or copper^{19~21)}.

From these points of view, the adsorption rates of mercuric and copper ions by oral bacteria derived from human dental plaque were examined.

In the adsorption of the mercuric ions at pH 4.0, the adsorption rates by *P. acnes*, *T. denticola* and *E. corrodens* were highest among all species tested. For copper adsorption at pH 4.0, *T. denticola*, *P. anaerobius* and *C. matruchotii* showed the highest rates in all species tested.

The adsorption of copper by agarose used as a

control was about 20% lower than that of the microorganisms (Figs. 5, 6). As the polysaccharide such as agarose had some metal-adsorbing ability, the true adsorption of metal by bacteria should be compared with that of agarose. The relationship between the metal concentration of residual copper ions in solution and the amounts of adsorbed copper ions by *P. acnes* was also investigated according to the Freundlich adsorption isotherm equation. The results obtained showed a linear relationship.

It has been observed that metal adsorption by microorganisms is largely dependent on the cell concentration at a given equilibrium concentration of mercury.

The adsorptions of copper ions by both the lyophilized and viable cells treated with or without heat were compared. The copper ion-adsorbing ability of the heat killed-cells was slightly less than that of the non-heated cells on viable *P. acnes* (Fig.2).

Also, the equilibration times for mercuric and copper ions with *p. acnes* were examined at pH 4.0. After the incubation of the microorganisms with the metal ions for 5 min, the adsorption rates of these metal ions by *P. acnes* reached a plateau.

These results suggest that there is no energy requirement for the adsorption of copper ions by *P. acnes* and that the accumulation of copper by the microorganisms depends on physico-chemical adsorption at the cell surface rather than on metabolic uptake.

The heavy metal-adsorbing abilities of the oral microorganisms have not been reported previously. The results obtained herein indicated that some species of oral microorganisms had high mercury and copper-adsorbing abilities.

It is recognized that dental plaque mainly consists of many kinds of oral microorganisms closely related to the etiology of dental caries and periodontal diseases. On the other hand, numerous oral microbes inhabit the oral cavity as well as endogenous microbes and matrix materials such as salivary components and carbohydrates.

Therefore, oral flora are also thought to disturb the establishment of exogenous microbes.

It may be assumed that the oral microflora may

play some role as a barrier to protect exogenous origins in the oral eco-system. From the results obtained, as some oral microorganisms have high metal ion-adsorbing abilities, dental plaque may serve to eliminate heavy metal ions from the oral cavity.

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